

METHODS FOR IDENTIFYING LIGAND SPECIFIC BINDING MOLECULES

This application claims the benefit of priority of United States Serial No. 08/905,825, filed August 4, 1997, which was converted to a United States Provisional
5 Application, the entire contents of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present invention relates generally to tumor cell therapy and more specifically to methods of
10 identifying new tumor specific binding molecules and tumor specific antigens.

Recent years have provided tremendous progress in the understanding of cancer development and progression. Despite this increased knowledge, only
15 marginal decreases in death rates from most types of cancer have been observed. The success rate of cancer therapy is increased with early diagnosis. Cancer therapy generally involves treatment with therapeutic agents that affect not only cancer cells but other cells
20 in the body as well, often leading to debilitating side effects. Thus, identification of tumor-specific targeting agents, such as antibodies that bind to tumor antigens, would provide reagents useful for earlier diagnosis of specific types of cancers as well as
25 tumor-specific targeting agents for cancer therapy that minimizes impact on non-tumor tissues.

Although some tumor-specific antigens have been identified, obtaining useful tumor-specific targeting agents has remained elusive. Of interest for therapeutic
30 purposes are human antibodies that can be used to target toxins to specific types of tumors. The advantage of using human antibodies is that they are least likely to cause an immune response that would remove the antibody and toxin from the body during cancer therapy. However,

development of human antibodies capable of targeting specific tumors has proven difficult.

To obtain antibodies or other agents capable of specifically targeting tumor types, a screen of a large number of possible agents is required. For example, to obtain a monoclonal antibody specific for a tumor antigen, it is necessary to generate and screen tens or hundreds of hybridoma cell lines. The process is laborious, time consuming and additionally requires the initial purification or preparation of the antigen or tumor cell.

A number of other screening approaches have now been developed, including recombinant methods utilizing bacteria and yeast, that allow identification of specific binding partners for a particular molecule of interest. For example, it is now possible to produce large display or combinatorial libraries of antibodies or other types of binding molecules. However, these methods generally require the screening of the libraries using one or more relatively purified molecules of interest.

Regardless of the available screening approaches, the identification of tumor specific antigens and their subsequent isolation has proven difficult over the years. Methods which have attempted to circumvent this problem through the use of cell lysates to screen for specific binding molecules has unfortunately resulted in molecules which exhibit inadequate binding specificities. Therefore, the availability of specific binding molecules which can be adapted to cancer diagnosis and therapy has been inherently lacking.

Thus, there exists a need for rapid and efficient methods to identify specific binding molecules

to tumor antigens. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides a method for
5 identifying a binding molecule having selective affinity
for a ligand. The method consists of selectively
immobilizing a diverse population of binding molecules to
a solid support, simultaneously contacting the diverse
population immobilized on the solid support with two or
10 more ligands and determining at least one binding
molecule which selectively binds to one or more of the
ligands. The invention additionally provides a method
for identifying an antibody having selective affinity for
a tumor antigen. The method consists of selectively
15 immobilizing a diverse population of antibodies to a
solid support, simultaneously contacting the diverse
population immobilized on the solid support with two or
more tumor antigens and determining at least one antibody
which selectively binds to one or more of the tumor
20 antigens. The invention also provides an isolated
binding polypeptide selective for a tumor antigen.
Further provided by the present invention is a
Complementarity Determining Region (CDR) or functional
fragment thereof of an antibody selective for a tumor
25 antigen.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the optimization of human Fab
binding molecule immobilization on a solid support.

Figure 2 shows the identification of human Fab
30 binding molecules to tumor antigens using binding

molecules selectively immobilized on a solid support and compares detection efficiencies to binding molecules directly immobilized on a solid support.

Figure 3 shows the increased binding
5 specificity and decreased background of detection using selective immobilization of human Fab binding molecule populations on a solid support compared to direct immobilization.

Figure 4 shows the binding specificity of human
10 Fab binding molecules identified by selective immobilization on a solid support to tumor cell monolayers.

Figure 5 shows fluorescent activated cell
15 sorting of tumor cells with human Fab binding molecules identified by selective immobilization on a solid support.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides rapid and efficient
methods for the identification of binding molecules which
20 exhibit selective affinity for one or more ligands of interest. The methods are advantageous in that they allow the simultaneous screening of multiple binding molecules against multiple ligands of interest. Moreover, very little information is required regarding
25 the identity or function of either the binding molecule or the ligand. For example, diverse populations of binding molecules can be simultaneously screened against diverse populations of ligands to rapidly identify numerous molecules exhibiting a desired binding
30 specificity. The methods of the invention can therefore

be advantageously applied for the discovery of specific reagents for diagnosis and treatment of human diseases.

As used herein, the term "binding molecule" is intended to refer to a molecule of sufficient size and complexity so as to be capable of selectively binding a ligand. Such molecules are generally macromolecules, such as polypeptides, nucleic acids, carbohydrate or lipid. However, derivatives, analogues and mimetic compounds as well as small organic compounds are also intended to be included within the definition of this term. The size of a binding molecule is not important so long as the molecule exhibits or can be made to exhibit selective binding activity to a ligand. For example, a binding molecule can be as little as about one or two, and as many as tens or hundreds of monomer building blocks which constitute a macromolecule binding molecule. Similarly, an organic compound can be a simple or complex structure so long as selective binding affinity can be exhibited.

Binding molecules can include, for example, antibodies and other receptor or ligand binding polypeptides of the immune system. Such other molecules of the immune system include for example, T cell receptors (TCR), major histocompatibility complex (MHC), CD4 receptor, and CD8 receptor. Additionally, cell surface receptors such as integrins, growth factor receptors and cytokine receptors, as well as cytoplasmic receptors such as steroid hormone receptors are substantially also included within the definition of the term binding molecule. Furthermore, DNA binding polypeptides such as transcription factors and DNA replication factors are likewise included within the definition of the term binding molecule. Finally,

polypeptides, nucleic acids and chemical compounds such as those selected from random and combinatorial libraries are also included within the definition of the term so long as such a molecule exhibits or can be made to exhibit selective binding activity toward a ligand.

As used herein, the term "polypeptide" when used in reference to a binding molecule or a ligand is intended to refer to peptide, polypeptide or protein of two or more amino acids. The term is similarly intended to refer to derivatives, analogues and functional mimetics thereof.

As used herein, the term "ligand" refers to a molecule that can be selectively bound by a binding molecule. A ligand can be essentially any type of molecule such as polypeptide, nucleic acid, carbohydrate, lipid, or any organic derived compound. Those skilled in the art know what is meant by the meaning of the term ligand. Specific examples of ligands are the tumor antigens described herein which are selectively bound by the human antibody binding molecules described in the examples.

As used herein, the term "diverse population" is intended to refer to a group of two or more different molecules. A diverse population of binding molecules can have similar biochemical function as long as the function or structure of the binding molecules are not identical. A diverse population can include, for example, a population of binding molecules that are antibodies capable of recognizing the same or different ligands. Moreover, the same binding molecule can recognize two different ligands based on different ligand conformations. In such cases, the binding molecules

would be considered distinct based on function and multiple molecules of the same binding molecule would therefore comprise a diverse population.

As used herein, the term "selective" or
5 "selectively" when referring to the binding of a binding molecule to a ligand or the immobilization of a population to a solid support is intended to mean that the interaction can be discriminated from unwanted or non-specific interactions. Discrimination can be based
10 on, for example, affinity or avidity and therefore can be derived from multiple low affinity interactions or a small number of high affinity interactions. For example, a binding molecule interaction with a ligand is generally greater than about 10^{-4} M, is preferably greater than
15 about 10^{-5} M and more preferably greater than about 10^{-6} M. High affinity interactions are generally greater than about 10^{-8} M to 10^{-9} M or greater.

As used herein, the term "immobilizing" or grammatical equivalents thereof, refers to the
20 attachment, as through the binding of a population of binding molecules, to a solid support. Immobilization can be through specific interactions with the binding molecule and an agent on the solid support. The agent can be, for example, a chemical moiety which allows
25 covalent or non-covalent interactions sufficient to hold the population of binding molecules to the solid support. Immobilization can also be through tethers or linkers. Such linkers can be covalent linkers, hydrolyzable linkers, photo-labile linkers or other linkers that allow
30 the binding molecules to be selectively attached. Linkers can also be polypeptides or other biomolecular linkers such as antibodies, lipid attachments, streptavidin, receptors, fusion polypeptides, or any

biomolecule that can tether the binding molecule to the solid support. Additionally, domains of polypeptides can similarly be linkers. For example, hydrophobic domains which allow direct absorption to a plastic due to
5 specific sequences which are molecular tags or recognition sequences can be linkers for binding polypeptides.

As used herein, the term "solid support" refers to a solid medium which is sufficiently stable so as to
10 allow immobilization of a population of binding molecules. Solid supports can include, for example, membranes such as nitrocellulose, nylon, polyvinylidene difluoride, plastic, glass, polyacrylamide or agarose. Solid supports can also be made in essentially any size
15 or shape so long as it supports the immobilization of a population of binding molecules. For example, the solid support can be a flat planar surface such as a natural or synthetic membrane filter or a glass slide. Alternatively, the solid support can be of various
20 spherical shapes, including, for example, beads made of glass, polyacrylamide or agarose. Porous mediums can similarly be used as solid supports and such mediums are included within the definition of the term as used herein. Additionally, any of the solid supports can be
25 modified, for example, to include functional chemical groups that can be used directly or indirectly for attachment of binding molecules or linkers.

As used herein, the term "antibody" refers to a polypeptide which binds to a ligand and is intended to be
30 used consistently with its meaning within the art. The term immunoglobulin is similarly intended to fall within the scope of the meaning of the term antibody as it is known and used within the art. The polypeptide can be

the entire antibody or it can be any functional fragment thereof which binds to the ligand. The meaning of the term is intended to include minor variations and modifications of the antibody so long as its function remains uncompromised. Functional fragments such as Fab, F(ab)₂, Fv, single chain Fv (scFv) and the like are similarly included within the definition of the term antibody. Such functional fragments are well known to those skilled in the art. Accordingly, the use of these terms in describing functional fragments of an antibody are intended to correspond to the definitions well known to those skilled in the art. Such terms are described in, for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1989); Molec. Biology and Biotechnology: A Comprehensive Desk Reference (Myers, R.A. (ed.), New York: VCH Publisher, Inc.); Huston et al., Cell Biophysics, 22:189-224 (1993); Plückthun and Skerra, Meth. Enzymol., 178:497-515 (1989) and in Day, E.D., Advanced Immunochimistry, Second Ed., Wiley-Liss, Inc., New York, NY (1990), which are incorporated herein by reference.

As with the above terms used for describing antibodies and functional fragments thereof, the use of terms which reference other antibody domains, functional fragments, regions, nucleotide and amino acid sequences and polypeptides or peptides, is similarly intended to fall within the scope of the meaning of each term as it is known and used within the art. Such terms include, for example, "heavy chain polypeptide" or "heavy chain", "light chain polypeptide" or "light chain", "heavy chain variable region" (V_H) and "light chain variable region" (V_L) as well as the term "complementarity determining region" (CDR).

In the case where there are two or more definitions of a term which is used and/or accepted within the art, the definition of the term as used herein is intended to include all such meanings unless

5 explicitly stated to the contrary. A specific example is the use of the term "CDR" to describe the non-contiguous antigen combining sites found within the variable region of both heavy and light chain polypeptides. This particular region has been described by Kabat et al.,

10 U.S. Dept. of Health and Human Services, "Sequences of Proteins of Immunological Interest" (1983) and by Chothia et al., J. Mol. Biol. 196:901-917 (1987) and additionally by MacCallum et al., J. Mol. Biol. 262:732-745 (1996), which are incorporated herein by reference, where the

15 definitions include overlapping or subsets of amino acid residues when compared against each other. Nevertheless, application of either definition to refer to a CDR of an antibody or variants thereof is intended to be within the scope of the term as defined and used herein. The

20 appropriate amino acid residues which encompass the CDRs as defined by each of the above cited references are set forth below in Table I as a comparison. The exact residue numbers which encompass a particular CDR will vary depending on the sequence and size of the CDR.

25 Those skilled in the art can routinely determine which residues comprise a particular CDR given the variable region amino acid sequence of the antibody.

As used herein, the term "tumor antigen" refers to a molecule that can be used as a marker to

30 discriminate or distinguish a tumor cell from a normal cell. Such discriminatory markers can manifest themselves in various ways such as preferential expression or unique cellular or subcellular localization in a tumor cell. A tumor cell for which such antigens

Table I: CDR Definitions

| | <u>Kabat</u> ¹ | <u>Chothia</u> ² | <u>MacCallum</u> ³ |
|-----------------------|---------------------------|-----------------------------|-------------------------------|
| V _H CDR1 | 31-35 | 26-32 | 30-35 |
| V _H CDR2 | 50-65 | 52-56 | 47-58 |
| 5 V _H CDR3 | 95-102 | 95-102 | 93-101 |
| V _L CDR1 | 24-34 | 24-34 | 30-36 |
| V _L CDR2 | 50-56 | 50-56 | 46-55 |
| V _L CDR3 | 89-97 | 89-97 | 89-96 |

¹ Residue numbering follows the nomenclature of Kabat et al., *supra*

² Residue numbering follows the nomenclature of Chothia et al., *supra*

³ Residue numbering follows the nomenclature of MacCallum et al., *supra*

15 preferentially mark can be any cell which undergoes abnormally frequent cell proliferation or unregulated survival. Such a cell can be a neoplastic cell which may or may not be fully characterized and classified as tumor or cancer cell.

20 The invention provides a method for identifying a binding molecule having selective affinity for a ligand. The method consists of: a) selectively immobilizing a diverse population of binding molecules to a solid support; b) simultaneously contacting the diverse
25 population immobilized on the solid support with two or more ligands; and c) determining at least one binding molecule which selectively binds to one or more of the ligands.

The methods of the invention employ the
30 selective immobilization of binding molecules to a solid

support and the screening of the immobilized binding molecules for selective binding interactions against two or more ligands of interest. Selective immobilization functions to increase the sensitivity of the binding interaction being measured since initial immobilization of a selected population of molecules onto a solid support reduces non-specific binding interactions with irrelevant molecules or contaminants which can be present in the reaction. For example, the difficulty of measuring selective binding interactions increases with the complexity of the binding assay as well as the number and diversity of different species within the binding reaction. The methods of the invention circumvent such difficulties since the selective immobilization of the binding molecule population substantially reduces the number of unwanted binding interactions through the removal of irrelevant molecules within the reaction.

Methods exist in the art for generating and screening populations of binding molecules against one or more purified ligands or targets of interest. However, when populations of binding molecules are screened against populations of ligands or complex compositions such as cells or cell extracts, the identification of specific binding events is rare. The methods of the invention allow for the efficient screening of binding molecule populations against ligand populations for the rapid identification of selective binding events. The methods of the invention provide improved sensitivity and specificity of detection through the selective immobilization of the binding molecule population on a solid support.

The methods of the invention are applicable for both large and small populations of binding molecules

which can be either substantially purified or enriched such as through separation of the population by affinity techniques. Alternatively, such diverse populations can be heterogeneous compositions such as those being
5 contained within a cell, cell extract or an *in vitro* derived synthesis. Selective immobilization of such populations provides sufficient enrichment of the population so as to be applicable for screening with two or more ligands of interest for selective binding
10 affinity.

The choice of the binding molecule population will depend on the type of binding molecule desired, the need and the ultimate intended use of the binding molecule. For example, populations of binding molecules
15 can be produced or derived from essentially any source so long as the population contains at least one binding molecule which exhibits selective binding affinity for the ligand or ligand composition of interest or so long as the population is of sufficient diversity so that
20 there is a very likely probability that the population will contain at least one molecule which will exhibit selective binding to the ligand of interest.

At least two general approaches can be used for obtaining binding molecule populations which will meet
25 the above criteria. One approach is to generate binding molecule populations from molecules known to function as binding molecules or known to exhibit or be capable of exhibiting binding activity. A specific example of molecules known to function as binding molecules or
30 exhibit binding activity include antibodies and other receptors of the immune repertoire. The normal function of such molecules is to bind essentially an infinite number of different antigens and ligands. Therefore,

generating a diverse population of binding molecules from an antibody repertoire, for example, will allow the identification of a binding molecule against essentially any desired ligand. Numerous other binding molecule
5 populations exist as well and are described further below.

A second approach is to generate a large population of unknown molecules. The population should be generated to contain a sufficient diversity of
10 sequence or structure so as to contain a molecule which will bind to the ligand composition of interest. An advantage of this approach is that no prior knowledge of sequence, structure or function is required. Instead, all that is necessary is to generate a population of
15 sufficient size and complexity so that the population will have a high probability of exhibiting a specific binding interaction to the ligand complex by chance. A specific example of such a population are random libraries of peptides, nucleic acids and small molecule
20 compounds. Numerous other types of "random" library molecule populations exist and are described further below.

Prior knowledge of whether populations obtained by either of the approaches described above contain at
25 least one binding molecule which exhibits selective binding affinity for the ligand or composition of interest is not necessary. Instead, all that is necessary is to produce a population sufficiently diverse for the intended need or purpose so as to likely contain
30 a binding molecule against the ligands or ligand compositions of interest. Those skilled in the art will know what size and diversity is necessary or sufficient for the intended purpose.

As stated previously, the choice of which type of binding molecule population to use will depend on the specific need and desired use of the selected binding molecule. For example, if binding molecules having high affinity interaction are desired, then the use of a population of antibody binding molecules can be preferred. Other binding polypeptides and receptors of the immunoglobulin superfamily of receptors additionally can be used as well. Alternatively, if binding molecules are desired for receptors, for example, it may be desired to use populations of random peptide or nucleic acid sequences. Specific examples would include, for example, the use of random peptide populations to identify peptide binding molecules specific for cell surface receptors, random small molecule populations for the identification of binding molecules having selective binding affinity to cytoplasmic receptors such as steroid receptors, and random nucleic acid populations for the identification of binding molecules selective for nucleic acid binding polypeptides. Those skilled in the art will know or can determine what type of approach and what type of binding molecule population is applicable for an intended purpose and desired need.

The size and diversity of the binding molecule population to use will depend on, for example, the ligand population or ligand composition for which selective binding molecules are desired as well as the number of selective binding molecules desired, the range of desired affinities and the time frame available for identifying an appropriate binding molecule. For example, if the application of the methods of the invention are for the identification of one or a few binding molecules with selective affinity for a ligand, then a limited number of binding molecules can comprise the population to be

selectively immobilized on a solid support. Such a limited number of binding molecules can be as small as between about two to ten but will typically comprise tens of molecules.

5 As the desired number of binding molecules to be identified increases, so does the size and diversity of the population. Similarly, as the range of desired affinities and speed of identification of binding molecules with selective affinity for a ligand increases,
10 so does the size and diversity of the binding molecule population. Moreover, the size of the population of binding molecules will similarly increase as the number of ligands or complexity of the ligand population increases for which selective binding molecules are
15 desired. Thus, as each of these factors increase, the size and diversity of the binding molecule population can similarly increase.

Moderate sized populations will consist of hundreds and thousands of different binding molecules
20 within the population whereas a large sized binding molecule population will consist of tens of thousands and millions of different binding molecule species. More specifically, large and diverse populations of binding molecules for the identification of a binding molecule
25 will contain any of about 10^4 , 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , or more, different binding molecule species. One skilled in the art will know the approximate diversity of the population of binding molecules which will be sufficient to identify the desired number of binding
30 molecules.

Recombinant libraries of binding molecules are particularly advantageous for the methods of the

invention since large and diverse populations can be rapidly generated and screened with two or more ligands of interest. Binding molecule populations can be directly used from the cell extracts of library
5 populations, for example. Methods employed for selectively immobilizing the binding molecule populations are discussed further below. Moreover, recombinant libraries of expressed polypeptide or nucleic acid products are applicable to the methods of the invention
10 since they can be engineered in a large number of different ways so as to facilitate or directly function in the selective immobilization of the binding molecule population to a solid support. Recombinant library methods similarly allow for the production of a large
15 number of binding molecule populations from naturally occurring repertoires which inherently contain features for selective immobilization of the population to a solid support. Nevertheless, whether recombinant or otherwise, essentially any source of binding molecule populations
20 can be used so long as the source provides a sufficient size and diversity of different binding molecules appropriate for the intended purpose and so long as the population of binding molecules can be selectively immobilized to a solid support.

25 Thus, the invention provides a method of identifying a binding molecule having selective affinity for a ligand wherein the binding molecule population is produced by a recombinant library. Binding molecule populations other than recombinant libraries exist as
30 well and can similarly be used for the screening and identification of binding molecules having selective affinity for a ligand.

A specific example of recombinant libraries as sources of binding molecule populations are phage expression libraries in which lysogenic phage cause the release of bacterially expressed binding molecule polypeptides. In another type of phage expression library, large numbers of potential binding molecules can be expressed as fusion polypeptides on the periplasmic surface of bacterial cells. In both of these examples, the expressed polypeptides are available for immobilization on a solid support. Libraries in yeast and higher eukaryotic cells exist as well and are similarly applicable in the methods of the invention. Those skilled in the art will know or can determine what type of library is applicable for a specific purpose.

The invention also provides a method for identifying a binding molecule selective for a ligand, wherein the binding molecule is identified from an antibody population.

As stated previously, there are at least several approaches from which to obtain a starting population of binding molecules to use for the identification of a molecule having selective binding affinity for a ligand. One approach is to use a population of binding molecules that normally exhibit binding function. Antibody binding molecules are amenable to such an approach since they inherently function as binding molecules and can be generated by biological and recombinant methods to exhibit binding specificities to essentially any antigen. Thus, the populations of binding molecules of the invention can be antibodies or functional fragments thereof.

Populations of antibody binding molecules can be generated by a variety of means known to those skilled in the art. For example, populations of antibody binding molecules can be generated from hybridoma technology and
5 populations of monoclonal antibodies can be used in the methods of the invention to identify a monoclonal antibody binding molecule, for example, having selective affinity for a ligand. Alternatively, large populations of antibody binding molecules can be expressed, for
10 example, by various recombinant methods known in the art.

Methods exist in the art to amplify, by polymerase chain reaction and other related methods known to those skilled in the art, essentially the entire antibody repertoire of a particular organism and express
15 as a recombinant population a diverse combination of the heavy and light antibody chains or functional fragments thereof. Organisms from which antibody repertoires can be derived include, for example, human, mouse, human, rabbit, goat, chicken or any organism capable of
20 producing antibodies. Functional fragments include, for example, Fab, Fv and CDR regions of the antibody molecules. The antibodies or functional fragments thereof can then be screened by selective immobilization onto a solid support. In the Examples described herein,
25 a human Fab fragment expression library was used to screen for binding molecules with selective affinity for tumor antigens.

In addition to antibody repertoires as starting sources for binding molecule populations having inherent
30 binding capabilities, the binding molecule populations can also be derived from other molecules of the immune system having known or inherent binding capabilities. For example, the binding molecules can be T cell

receptors (TCR). T cell receptors contain two subunits, α and β , which are similar to antibody variable region sequences in both structure and function. In this regard, both subunits contain variable region which
5 encode CDR regions similar to those found in antibodies (Immunology, Third Ed., Kuby, J. (ed.), New York, W.H. Freeman & Co. (1997), which is incorporated herein by reference). The CDR containing variable regions of TCRs bind to antigens presented on the cell surface of
10 antigen-presenting cells and are capable of exhibiting binding specificities to essentially any particular antigen. As with antibody repertoires, there is sufficient TCR sequence information known to allow the amplification and recombinant engineering of essentially
15 the entire TCR repertoire. Thus, TCRs are amenable to the methods of the invention since they inherently function as binding molecules and can be generated by biological and recombinant methods to exhibit binding specificities to essentially any antigen.

20 Other exemplary binding molecules of the immune system which exhibit known or inherent binding functions include receptors of the major histocompatibility complex (MHC), CD4 and CD8. MHC functions in mediating interactions between antigen-presenting cells and
25 effector T cells. CD4 and CD8 receptors function in binding interactions between effector T cells and antigen-presenting cells. CD4 and CD8 have the additional advantage in that they exhibit similar CDR region structure as do antibodies and TCRs sequences.

30 Other binding molecules exhibiting known or inherent binding function which are amenable for use as starting populations in the methods of the invention include a variety of receptors such as cell surface,

cytoplasmic and nuclear receptors. Specific example of each of these classes of binding molecules will be provided below. Binding molecules other than those discussed below also exist and can be used in the methods
5 of the invention. Such other molecules are known or can be determined by those skilled in the art using the teachings provided herein.

Integrins, for example, are cell surface receptors involved in a variety of physiological
10 processes such as cell attachment, cell migration and cell proliferation. Integrins mediate both cell-cell and cell-extracellular matrix adhesion events. Structurally, integrins consist of heterodimeric polypeptides where a single α chain polypeptide noncovalently associates with
15 a single β chain. In general, different binding specificities are derived from unique combinations of distinct α and β chain polypeptides. For example, vitronectin binding integrins contain the α_v integrin subunit and include $\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$, all of which
20 exhibit different ligand binding specificities.

Binding molecule populations can also be produced from other cell surface receptors. These other cell surface receptors include growth factor or hormone receptors for ligands such as platelet derived growth
25 factor, epidermal growth factor, insulin, insulin-like growth factor, hepatocyte growth factor, and other growth factors and hormones. In addition, cell surface receptors include cytokine receptors such as those for the interleukins and interferons.

30 Cytoplasmic receptors can additionally be used as the binding molecule population for use in the methods

of the invention. Cytoplasmic receptors include, for example, the steroid hormone receptors such as the thyroid hormone receptor, retinoic acid receptor, glucocorticoid receptor, estrogen receptor and others.

- 5 In addition, binding molecule populations can be generated from DNA or other nucleic acid binding polypeptides that exhibit known or inherent binding function in processes such as replication, transcription, processing or translation.

- 10 As described previously, the binding molecule populations can also be derived from random libraries of unknown sequences or structures. In this approach it is sufficient to produce a large and diverse library so as to exhibit a likely probability that one or more
- 15 sequences within the binding molecule population will exhibit selective binding affinity to the ligand of interest. All that is necessary to practice the methods of the invention using such populations is to be able to selectively immobilize the random population of binding
- 20 molecules to a solid support. Methods are described below for selectively immobilizing essentially any population of binding molecules to a solid support. Moreover, using the teachings described herein, those skilled in the art will know how to apply other methods
- 25 so as to allow the selective immobilization of a population of binding molecules to a solid support.

- Finally, the binding molecules can also be synthetic compounds produced by, for example, combinatorial chemistry methods known to those skilled in
- 30 the art. Such combinatorial chemical libraries can be selectively immobilized onto a solid support and then screened for the identification of molecules having selective binding affinity for a ligand. Additionally,

large populations of other compounds, such as natural or synthetic compound libraries are also equally applicable as a binding molecule population so long as they can be selectively immobilized onto a solid support.

5 The invention provides a method for identifying a binding molecule exhibiting selective affinity for a ligand from a diverse population of binding molecules. The binding molecule is identified by simultaneously contacting a diverse population of immobilized binding
10 molecules with two or more ligands.

 The methods of the invention allow for the simultaneous screening of two or more ligands of interest. Binding molecules exhibiting selective affinity can be obtained to one or more of the ligands of
15 interest. The ligand population will be selected depending on the need and intended use of the binding molecule as well as the characteristics of the ligands or ligand compositions. For example, using the methods of the invention to selectively immobilize a population of
20 binding molecules, selective binders can be identified to ligands as complex as tumor cells or specific tissue types as well as to simple ligand populations of two or more different species. An advantage of the method is that sources of relatively crude ligand populations can
25 be used which facilitates the use of whole tissues, tumor cells and cell lysates as ligand sources. In addition, ligands which are not biochemically well characterized can additionally be used in the methods of the invention.

 In the case where the ligand populations are
30 complex compositions such as tissues, cells, cell lysates, and cellular or subcellular compartments, it is the individual molecules which comprise each of these

complex compositions that make up the ligand population. Therefore, by choosing a complex composition such as a cell or cell lysate, for example, the ligand population inherently contains two or more ligands that are used to
5 screen a population of binding molecules immobilized on a solid support. Identifying a binding molecule selective to at least one of the ligands within the population will yield a binding molecule selective to the complex composition itself. Thus, the methods of the invention
10 are applicable to the efficient identification of binding molecules to, for example, tumor cells or other cellular pathologies for the selective targeting of therapeutic agents. The methods of the invention are similarly applicable to the identification of binding molecules to
15 normal or diseased tissue for the selective targeting of, for example, diagnostic agents such as imaging reagents.

In order to identify a binding polypeptide having selective binding affinity to the ligand, it is necessary to selectively immobilize a population of
20 binding molecules and then to contact the binding molecules with two or more ligands. As described further below, successful binding of a binding molecule within the population indicates selective binding to at least one of the ligands within the population. Selectivity
25 can be further confirmed by, for example, comparing the binding affinity of the positive binder to a non-specific or control ligand population. If desired, such a comparison can be performed as a control during the initial identification of the binding molecule such as
30 using normal cells and tumor cells as the control and ligand populations, respectively. Alternatively, such a comparison can be subsequently performed using either the initial ligand populations or more purified form of either the binding molecule or the ligand of interest.

Thus, the invention provides a method of identifying a binding molecule having selective affinity for a ligand wherein the ligand is a tissue or tumor antigen. The invention also provides a method of
5 identifying a binding molecule having selective affinity for a ligand wherein the ligand is a polypeptide or other macromolecule in a cell lysate.

In addition to ligand populations derived from crude cell preparations or other complex compositions,
10 the ligand populations can also be derived from simple populations of substantially purified molecules. For example, if it is desired to produce a binding molecule selective for any one member of a population of ligands, then each individual member can be combined into a single
15 population and screened simultaneously using the methods of the invention. The molecules can be substantially purified or contain various amounts of other irrelevant species. Additionally, in cases such as this, where a binding molecule is desired which is selective for one or
20 a few members of a population, if the individual ligand species can be produced by recombinant or other biological synthetic mechanisms, then cells can be engineered to express the ligands. The ligands can then be screened in crude cell extracts to identify a binding
25 molecule which is selective for at least one ligand in the population. Such cells can be engineered to synthesize each ligand individually and then cell extracts can be pooled for screening. Alternatively, a cell can be engineered to express multiple ligands so
30 that only one or a few cell extracts are required to be combined and screened.

The ligand populations can be composed of different sizes of either substantially purified

molecules or crude cell preparations or other complex compositions. Generally, simple populations of ligands contain at least two different ligand species, and can be composed of any of 3, 4, 5, 6, 7, 8, 9 or 10 different
5 ligands. Moderate ligand populations contain between about ten and several hundreds of different ligand species. Complex ligand populations contain about tens of thousands of different ligand species including, for example, the number of different molecules within a cell.
10 This number is generally thought to be about 10^5 . Even further, recombinant libraries and combinatorial libraries can be screened which can be composed of ligand populations of 10^6 , 10^7 , 10^8 and as large as greater than 10^{10} different species. Each of these population sizes
15 can be used as a ligand population for which to identify a binding molecule having selective binding affinity. The choice of the population size and type will depend on the need and intended use of the binding molecule. One skilled in the art will know which size and type of
20 population is suitable for a particular need.

As stated previously, the ligands which comprise the above described populations can be essentially any type of molecule such as a polypeptide, nucleic acid, carbohydrate, lipid or any organic
25 compound. Therefore, it is understood that molecules discussed above as binding molecules can also be ligands. For example, an individual or group of cell surface receptors such as integrins, growth factor receptors or cytokine receptors can be used as ligands to screen a
30 random peptide library or combinatorial chemical library to identify binding molecules that could be used as agonists or antagonists for these receptors.

To conduct a screen for binding molecules which bind to a ligand or group of ligands, the ligands themselves are detected by an appropriate detection method. The detection method can be direct or indirect and can involve detection of, for example, light emission, radioisotopes, color development, or any method which allows the ligand to be detected. Direct detection can involve methods such as radioactive labeling of ligands using metabolic labeling in an appropriate cell or *in vitro* labeling using *in vitro* coupled transcription-translation with appropriate radioactive amino acids, covalent modification with a radioactive substrate using an appropriate enzyme such as a kinase, or chemical modification using radioisotopes such as iodination. Direct methods can also involve fusion of an appropriate detection molecule to the ligand. For example, the ligand can be fused to luciferase and detected by light emission or can be fused to lacZ and detected by appropriate calorimetric detection. In the case where a population of ligands is desired to have biologically derived detection moieties, a library containing ligands fused to such detectable molecules as luciferase and lacZ can be used to screen for binding molecules. Direct detection of a ligand can also be performed by covalent modification of the ligand by incorporating a chemical moiety capable of being detected. For example, biotin can be covalently attached to the ligand and subsequently detected by streptavidin using one of the detection methods discussed above.

For indirect detection of ligands, a molecule that is not covalently attached to the ligand can be used for detection. A molecule known to interact with the ligand is used for such indirect detection methods. For example, a known molecule such as an antibody capable of

specifically detecting the ligand can be employed. The known molecule is either itself detected by one of the methods described above for direct detection of the ligand or is detected by a secondary molecule specific
5 for the first known molecule. For example, an antibody specific for a ligand can be detected using a secondary antibody capable of interacting with the first antibody specific for the ligand, again using the detection methods described above for direct detection.

10 In one embodiment, ligands are derived from the cell surface of tumor cells. Cell surface molecules can be labeled, for example, with a detectable moiety such as a radioisotope or biotin. This labeling provides a source of ligand where the only characteristic that needs
15 to be known is that it is on the surface of a cell. As described in the Examples, tumor antigen ligands were prepared by labeling cell surface polypeptides of breast carcinoma cells with biotin and used to identify Fab fragment binding molecules selective for the tumor cell
20 surface polypeptides. Ligand populations derived from other cell or subcellular compartments can similarly be used in the methods of the invention to obtain a binding molecule that exhibits selective affinity for at least one ligand within the initial population. Thus, the
25 methods of the invention are applicable to a large variety of ligand populations in which selective binding affinity is required for the therapeutic treatment or diagnosis of a disease.

The methods of the invention consist of
30 selectively immobilizing a diverse population of binding molecules to a solid support. For selective immobilization, either an inherent characteristic of the binding molecules which comprise the population is

exploited to provide selective immobilization or, alternatively, the molecules are engineered to contain a specific characteristic to be used for selective immobilization. For example, a binding molecule itself
5 may contain a hydrophobic chemical group or domain or may be fused to a hydrophobic chemical group or domain that causes the binding molecule to be immobilized to a hydrophobic solid support such as plastic. In another example, the solid support can be coated with a chemical
10 moiety or a biomolecule such that it is able to bind to and selectively immobilize only the binding molecules that make up the binding molecule population. For example, the solid support can be coated with a biomolecule that selectively binds to a domain or
15 sequence that is common to the binding molecules. The use of such biomolecules as linkers or tethers should be selected so that they do not interfere with the ligand binding to the binding molecules. For example, an antibody against the constant region of an antibody can
20 be used to selectively immobilize antibody binding molecules. Such an approach was used to immobilize human Fab fragments with anti-human kappa antibodies as described in the Examples. Alternatively, antibodies specific to peptide tags similarly function to
25 selectively immobilize a binding molecule population onto a solid support.

One advantage of selective immobilization is that it provides increased sensitivity and decreased non-specific binding for detecting specific binding
30 molecules of interest. Solid supports can, for example, immobilize a high concentration of binding molecules. Such high concentrations of binding molecules can serve to increase the ability to detect low affinity interactions with ligands and to detect ligands in low

concentrations. Essentially any solid support is amenable for use in the methods of the invention. The support should be chosen to fit a particular need and to exhibit the capacity to immobilize all, or a
5 statistically representative number of binding molecules of a population. In addition, solid supports can be made of porous materials which allow greater densities of immobilized binding molecules to be achieved.

Solid supports can also be chosen to increase
10 manipulability of a desired screen while maintaining the ability to retain the binding molecule population. Such manipulations can be important for removing unbound ligand populations and for washing to remove non-specific interactions. The ease of manipulation can be an
15 advantage when performing high throughput screening of a large number of binding molecules.

To perform the methods of the invention, once the population of binding molecules are immobilized on a solid support, the immobilized population is
20 simultaneously contacted with a population of two or more ligands. The population of ligands is incubated with the selectively immobilized binding molecules under appropriate conditions for an appropriate period of time so as to allow binding of the ligands to the binding
25 molecules. A binding molecule having selective affinity is identified by determining the binding of at least one ligand within the population to at least one binding molecule. One skilled in the art will know the appropriate conditions and can readily determine
30 conditions sufficient to allow interactions between binding molecules and ligands so as to detect the selective binding of a binding molecule to a ligand, thus

allowing the identification of a binding molecule having selective affinity for a ligand.

In addition, the invention provides methods for determining the sequence of identified binding molecules.

5 For example, if the binding molecules are produced in an expression library, encoding nucleic acids can be isolated from selected clones expressing binding molecules identified in a screen. The encoding nucleic acids can then be sequenced using methods known to those

10 skilled in the art.

Additionally, the invention provides a method for characterizing a ligand which is selectively bound by a binding molecule. Once a binding molecule has been identified which is selective for a ligand, the ligand

15 can then be isolated by, for example, affinity methods known to those skilled in the art, and characterized. This characterization can be beneficial if the ligands used in the screen are not well characterized. Characterization of the ligand includes such techniques

20 as determining its apparent molecular weight by gel electrophoresis. Other methods applicable for characterizing ligands include, for example, high performance liquid chromatography (HPLC), mass spectrometry, or other methods that provide information

25 about the physical, biochemical or functional properties of the ligand. One skilled in the art will know appropriate methods for such characterization of ligands.

The invention additionally provides a method for identifying an antibody having selective affinity for

30 a tumor antigen. The method consists of: a) selectively immobilizing a diverse population of antibodies to a solid support; b) simultaneously contacting the diverse

population immobilized on the solid support with two or more tumor antigens; and c) determining at least one antibody which selectively binds to one or more of the tumor antigens.

5 The methods of the invention can be practiced using antibody and tumor cells as the binding molecules and ligand populations, respectively. The population of antibody binding molecules can be produced, for example, in an expression library. Labeling of cell surface
10 molecules of a tumor cell provides a population of detectable tumor antigen ligands.

 Using the methods of the invention, the antibody binding molecules are selectively immobilized to a solid support by, for example, the use of antibodies
15 which are specific to the constant region of antibody binding molecules or specific to a particular isotype or antibody subunit. For example, anti-kappa chain antibodies can be used to immobilize intact antibodies as well as Fab fragments to a solid support.

20 Antibody binding molecules immobilized to a solid support can be incubated with tumor antigens under appropriate conditions so as to allow the tumor antigens to bind to the antibody binding molecules. The antigens can be applied as whole cells, cell extracts or as
25 specific cellular fractions. After an appropriate period of time, unbound tumor antigens are removed from the solid support and selective binding is detected as previously described. One skilled in the art will know what conditions will allow binding of tumor cell ligands
30 to antibodies and can readily determine the conditions sufficient to allow interactions to allow identification of antibodies specific for tumor antigens. Tumor

antigens are detected to determine one or more antibody binding molecules which specifically interact with tumor antigens. The selectivity of an antibody binding molecule for a tumor antigen can be determined relative to a normal cell which is used as a control. Thus, a method of the invention provides a means to identify tumor antigen-specific antibody binding molecules which can be used for diagnostic or therapeutic purposes.

Numerous antibody binding molecules selective for tumor specific ligands have been identified by the methods of the invention. These antibody binding molecules are described below in the Examples. Moreover, the nucleotide sequences have been determined for the CDR regions of these tumor selective antibody binding molecules. Such encoding nucleic acids are similarly described below in the Examples. Thus, the invention provides a method of identifying a population of human antibodies or functional fragments thereof.

Also provided herein is an isolated binding polypeptide selective for a tumor antigen. The isolated binding polypeptide is an antibody fragment selected from the group of antibody fragments consisting of F3, F13, F14, F15, F19, F21, F22, F23, F26, F30, F31, F32, F33, F34, F35, F36, F37, F38, F40, F41, F42, F46, F49, F50, F52, F54, F55, F58, F63, F66, F67, F68, F69, F70, F72, F74, F76, F78, F79, F80, F81, F84, F85, F86, F93, F99, F104, F111, F112, F118, F126, F129, F130, F132, F133, F134, F135, F136, F138, F151, F158, F160, F174, F176, F177, F184, F186, F191, F197, F200, F202, F203, F207, F208, F212, F214, F217, F224, F231, F236, F238, TA50 and TA73. Such an isolated binding polypeptide selective for a tumor antigen can be, for example, an F3, F14, F15,

F19, F21, F22, F23, F26, F133, TA50 or TA73 antibody fragment.

A particularly useful isolated binding polypeptide selective for a tumor antigen can be an antibody fragment having an amino acid sequence including three particular light chain CDR sequences and three particular heavy chain CDR sequences. An isolated antibody fragment selective for a tumor antigen can have, for example, an amino acid sequence including the three light chain CDR sequences SEQ ID NO:6, SEQ ID NO:28 and SEQ ID NO:50 and the three heavy chain CDR sequences SEQ ID NO:72, SEQ ID NO:94 and SEQ ID NO:116 found in F3, or can have an amino acid sequence in which one or more of these CDRs are replaced by a substantially similar or unrelated CDR. From the above description, one skilled in the art understands that an isolated antibody fragment of the invention can have any sub-combination of the six F3 CDRs, such as any two, three, four or five F3 CDRs, or can have a single F3 CDR. An isolated antibody fragment selective for a tumor antigen also can have an amino acid sequence including the three light chain CDR sequences SEQ ID NO:8, SEQ ID NO:30 and SEQ ID NO:52 and the three heavy chain CDR sequences SEQ ID NO:74, SEQ ID NO:96 and SEQ ID NO:118 found in F14, or can have an amino acid sequence in which one or more of these CDRs are replaced by a substantially similar or unrelated CDR. From the above description, one skilled in the art understands that an isolated antibody fragment of the invention can have any sub-combination of the six F14 CDRs, such as any two, three, four or five F14 CDRs, or can have a single F14 CDR.

In another embodiment, an isolated antibody fragment selective for a tumor antigen can have an amino

acid sequence including the three light chain CDR sequences SEQ ID NO:10, SEQ ID NO:32 and SEQ ID NO:54 and the three heavy chain CDR sequences SEQ ID NO:76, SEQ ID NO:98 and SEQ ID NO:120 found in F15, or can have an amino acid sequence in which one or more of these CDRs are replaced by a substantially similar or unrelated CDR. From the above description, one skilled in the art understands that an isolated antibody fragment of the invention can have any sub-combination of the six F15 CDRs, such as any two, three, four or five F15 CDRs, or can have a single F15 CDR. In another embodiment, an isolated antibody fragment selective for a tumor antigen can have an amino acid sequence including the three light chain CDR sequences SEQ ID NO:12, SEQ ID NO:34 and SEQ ID NO:56 and the three heavy chain CDR sequences SEQ ID NO:78, SEQ ID NO:100 and SEQ ID NO:122 found in F19, or can have an amino acid sequence in which one or more of these CDRs are replaced by a substantially similar or unrelated CDR. From the above description, one skilled in the art understands that an isolated antibody fragment of the invention can have any sub-combination of the six F19 CDRs, such as any two, three, four or five F19 CDRs, or can have a single F19 CDR.

In another embodiment, an isolated antibody fragment selective for a tumor antigen can have an amino acid sequence including the three light chain CDR sequences SEQ ID NO:14, SEQ ID NO:36 and SEQ ID NO:58 and the three heavy chain CDR sequences SEQ ID NO:80, SEQ ID NO:102 and SEQ ID NO:124 found in F21, or can have an amino acid sequence in which one or more of these CDRs are replaced by a substantially similar or unrelated CDR. From the above description, one skilled in the art understands that an isolated antibody fragment of the invention can have any sub-combination of the six F21

CDRs, such as any two, three, four or five F21 CDRs, or can have a single F21 CDR. In another embodiment, an isolated antibody fragment selective for a tumor antigen can have an amino acid sequence including the three light chain CDR sequences SEQ ID NO:16, SEQ ID NO:38 and SEQ ID NO:60 and the three heavy chain CDR sequences SEQ ID NO:82, SEQ ID NO:104 and SEQ ID NO:126 found in F22, or can have an amino acid sequence in which one or more of these CDRs are replaced by a substantially similar or unrelated CDR. From the above description, one skilled in the art understands that an isolated antibody fragment of the invention can have any sub-combination of the six F22 CDRs, such as any two, three, four or five F22 CDRs, or can have a single F22 CDR.

15 In another embodiment, an isolated antibody fragment selective for a tumor antigen can have an amino acid sequence including the three light chain CDR sequences SEQ ID NO:18, SEQ ID NO:40 and SEQ ID NO:62 and the three heavy chain CDR sequences SEQ ID NO:84, SEQ ID NO:106 and SEQ ID NO:128 found in F23, or can have an amino acid sequence in which one or more of these CDRs are replaced by a substantially similar or unrelated CDR. From the above description, one skilled in the art understands that an isolated antibody fragment of the invention can have any sub-combination of the six F23 CDRs, such as any two, three, four or five F23 CDRs, or can have a single F23 CDR. In another embodiment, an isolated antibody fragment selective for a tumor antigen can have an amino acid sequence including and the three light chain CDR sequences SEQ ID NO:20, SEQ ID NO:42 and SEQ ID NO:64 and the three heavy chain CDR sequences SEQ ID NO:86, SEQ ID NO:108 and SEQ ID NO:130 found in F26, or can have an amino acid sequence in which one or more of these CDRs are replaced by a substantially similar or

unrelated CDR. From the above description, one skilled in the art understands that an isolated antibody fragment of the invention can have any sub-combination of the six F26 CDRs, such as any two, three, four or five F26 CDRs,
5 or can have a single F26 CDR.

In another embodiment, an isolated antibody fragment selective for a tumor antigen can have an amino acid sequence including and the three light chain CDR sequences SEQ ID NO:22, SEQ ID NO:44 and SEQ ID NO:66 and
10 the three heavy chain CDR sequences SEQ ID NO:88, SEQ ID NO:110 and SEQ ID NO:132 found in F133, or can have an amino acid sequence in which one or more of these CDRs are replaced by a substantially similar or unrelated CDR. From the above description, one skilled in the art
15 understands that an isolated antibody fragment of the invention can have any sub-combination of the six F133 CDRs, such as any two, three, four or five F133 CDRs, or can have a single F133 CDR.

In another embodiment, an isolated antibody
20 fragment selective for a tumor antigen can have an amino acid sequence including and the three light chain CDR sequences SEQ ID NO:24, SEQ ID NO:46 and SEQ ID NO:68 and the three heavy chain CDR sequences SEQ ID NO:90, SEQ ID NO:112 and SEQ ID NO:134 found in TA50, or can have an
25 amino acid sequence in which one or more of these CDRs are replaced by a substantially similar or unrelated CDR. From the above description, one skilled in the art understands that an isolated antibody fragment of the invention can have any sub-combination of the six TA50
30 CDRs, such as any two, three, four or five TA50 CDRs, or can have a single TA50 CDR.

In still another embodiment, an isolated antibody fragment selective for a tumor antigen can have an amino acid sequence including and the three light chain CDR sequences SEQ ID NO:26, SEQ ID NO:48 and SEQ ID NO:70 and the three heavy chain CDR sequences SEQ ID NO:92, SEQ ID NO:114 and SEQ ID NO:136 found in TA73, or can have an amino acid sequence in which one or more of these CDRs are replaced by a substantially similar or unrelated CDR. From the above description, one skilled in the art understands that an isolated antibody fragment of the invention can have any sub-combination of the six TA73 CDRs, such as any two, three, four or five TA73 CDRs, or can have a single TA73 CDR.

Additionally provided is a CDR or functional fragment thereof of an antibody selective for a tumor antigen, where the CDR is derived from an antibody fragment selected from the group consisting of F3, F13, F14, F15, F19, F21, F22, F23, F26, F30, F31, F32, F33, F34, F35, F36, F37, F38, F40, F41, F42, F46, F49, F50, F52, F54, F55, F58, F63, F66, F67, F68, F69, F70, F72, F74, F76, F78, F79, F80, F81, F84, F85, F86, F93, F99, F104, F111, F112, F118, F126, F129, F130, F132, F133, F134, F135, F136, F138, F151, F158, F160, F174, F176, F177, F184, F186, F191, F197, F200, F202, F203, F207, F208, F212, F214, F217, F224, F231, F236, F238, TA50 and TA73. Such a CDR can have, for example, an amino acid sequence that is substantially similar or identical to the CDR-encoding portion of an amino acid sequence encoding one of these antibody fragments.

The present invention also provides an isolated nucleic acid molecule encoding an antibody fragment of the invention. Such a nucleic acid molecule includes a nucleotide sequence encoding substantially the amino acid

sequence referenced as SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:52, SEQ ID NO:54, SEQ ID NO:56, SEQ ID NO:58, SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66, SEQ ID NO:68, SEQ ID NO:70, SEQ ID NO:72, SEQ ID NO:74, SEQ ID NO:76, SEQ ID NO:78, SEQ ID NO:80, SEQ ID NO:82, SEQ ID NO:84, SEQ ID NO:86, SEQ ID NO:88, SEQ ID NO:90, SEQ ID NO:92, SEQ ID NO:94, SEQ ID NO:96, SEQ ID NO:98, SEQ ID NO:100, SEQ ID NO:102, SEQ ID NO:104, SEQ ID NO:106, SEQ ID NO:108, SEQ ID NO:110, SEQ ID NO:112, SEQ ID NO:114, SEQ ID NO:116, SEQ ID NO:118, SEQ ID NO:120, SEQ ID NO:122, SEQ ID NO:124, SEQ ID NO:126, SEQ ID NO:128, SEQ ID NO:130, SEQ ID NO:132, SEQ ID NO:134, or SEQ ID NO:136.

A nucleic acid molecule encoding an antibody fragment can include, for example, the nucleotide sequence referenced as SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:55, SEQ ID NO:57, SEQ ID NO:59, SEQ ID NO:61, SEQ ID NO:63, SEQ ID NO:65, SEQ ID NO:67, SEQ ID NO:69, SEQ ID NO:71, SEQ ID NO:73, SEQ ID NO:75, SEQ ID NO:77, SEQ ID NO:79, SEQ ID NO:81, SEQ ID NO:83, SEQ ID NO:85, SEQ ID NO:87, SEQ ID NO:89, SEQ ID NO:91, SEQ ID NO:93, SEQ ID NO:95, SEQ ID NO:97, SEQ ID NO:99, SEQ ID NO:101, SEQ ID NO:103, SEQ ID NO:105, SEQ ID NO:107, SEQ ID NO:109, SEQ ID NO:111, SEQ

ID NO:113, SEQ ID NO:115, SEQ ID NO:117, SEQ ID NO:119, SEQ ID NO:121, SEQ ID NO:123, SEQ ID NO:125, SEQ ID NO:127, SEQ ID NO:129, SEQ ID NO:131, SEQ ID NO:133, or SEQ ID NO:135.

5 In one embodiment, a CDR or functional fragment thereof of an antibody selective for a tumor antigen can be derived from an F3, F14, F15, F19, F21, F22, F23, F26, F133, TA50 or TA73 antibody fragment. Such a CDR can include substantially the amino acid sequence referenced

10 as SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44,

15 SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:52, SEQ ID NO:54, SEQ ID NO:56, SEQ ID NO:58, SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66, SEQ ID NO:68, SEQ ID NO:70, SEQ ID NO:72, SEQ ID NO:74, SEQ ID NO:76, SEQ ID NO:78, SEQ ID NO:80, SEQ ID NO:82, SEQ ID NO:84,

20 SEQ ID NO:86, SEQ ID NO:88, SEQ ID NO:90, SEQ ID NO:92, SEQ ID NO:94, SEQ ID NO:96, SEQ ID NO:98, SEQ ID NO:100, SEQ ID NO:102, SEQ ID NO:104, SEQ ID NO:106, SEQ ID NO:108, SEQ ID NO:110, SEQ ID NO:112, SEQ ID NO:114, SEQ ID NO:116, SEQ ID NO:118, SEQ ID NO:120, SEQ ID NO:122,

25 SEQ ID NO:124, SEQ ID NO:126, SEQ ID NO:128, SEQ ID NO:130, SEQ ID NO:132, SEQ ID NO:134, or SEQ ID NO:136.

The present invention also provides an isolated nucleic acid molecule encoding a CDR of the invention. Such a nucleic acid molecule includes a nucleotide

30 sequence encoding substantially the amino acid sequence referenced as SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ

ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ
 ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ
 ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ
 ID NO:52, SEQ ID NO:54, SEQ ID NO:56, SEQ ID NO:58, SEQ
 5 ID NO:60, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66, SEQ
 ID NO:68, SEQ ID NO:70, SEQ ID NO:72, SEQ ID NO:74, SEQ
 ID NO:76, SEQ ID NO:78, SEQ ID NO:80, SEQ ID NO:82, SEQ
 ID NO:84, SEQ ID NO:86, SEQ ID NO:88, SEQ ID NO:90, SEQ
 ID NO:92, SEQ ID NO:94, SEQ ID NO:96, SEQ ID NO:98, SEQ
 10 ID NO:100, SEQ ID NO:102, SEQ ID NO:104, SEQ ID NO:106,
 SEQ ID NO:108, SEQ ID NO:110, SEQ ID NO:112, SEQ ID
 NO:114, SEQ ID NO:116, SEQ ID NO:118, SEQ ID NO:120, SEQ
 ID NO:122, SEQ ID NO:124, SEQ ID NO:126, SEQ ID NO:128,
 SEQ ID NO:130, SEQ ID NO:132, SEQ ID NO:134, or SEQ ID
 15 NO:136.

A nucleic acid molecule encoding a CDR can
 include, for example, the nucleotide sequence referenced
 as SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11,
 SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19,
 20 SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27,
 SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35,
 SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:43,
 SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:51,
 SEQ ID NO:53, SEQ ID NO:55, SEQ ID NO:57, SEQ ID NO:59,
 25 SEQ ID NO:61, SEQ ID NO:63, SEQ ID NO:65, SEQ ID NO:67,
 SEQ ID NO:69, SEQ ID NO:71, SEQ ID NO:73, SEQ ID NO:75,
 SEQ ID NO:77, SEQ ID NO:79, SEQ ID NO:81, SEQ ID NO:83,
 SEQ ID NO:85, SEQ ID NO:87, SEQ ID NO:89, SEQ ID NO:91,
 SEQ ID NO:93, SEQ ID NO:95, SEQ ID NO:97, SEQ ID NO:99,
 30 SEQ ID NO:101, SEQ ID NO:103, SEQ ID NO:105, SEQ ID
 NO:107, SEQ ID NO:109, SEQ ID NO:111, SEQ ID NO:113, SEQ
 ID NO:115, SEQ ID NO:117, SEQ ID NO:119, SEQ ID NO:121,
 SEQ ID NO:123, SEQ ID NO:125, SEQ ID NO:127, SEQ ID
 NO:129, SEQ ID NO:131, SEQ ID NO:133, or SEQ ID NO:135.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also included within the definition of the invention provided herein.

- 5 Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Preparation of Solid Supports Selectively Immobilizing Human Fab Fragment Populations

- 10 This example describes a method for preparing a filter for selectively immobilizing a population of binding molecules.

- The population of binding molecules selectively immobilized in this example were recombinantly expressed human Fab fragments. In this case, the human BR96 Fab fragment, which is a chimeric antibody reactive with a tumor-associated cell surface antigen, was used to determine conditions for binding human Fab fragments. The BR96 antibody is reactive with a Lewis Y-related carbohydrate (LeY antigen) predominantly associated with lamp-1, an integral membrane protein normally localized in lysosomes. The population of human Fab fragments was immobilized on a solid support by coating nitrocellulose filters with anti-human kappa antibody. The anti-human kappa antibody was used as an agent for attachment to the filters since they are specific for the antibody population and can therefore be used to selectively immobilize or "capture" the population onto the filters.
- 15
20
25

- Initially, conditions were determined for optimizing the amount of antibody that could be selectively immobilized on nitrocellulose filters. A variety of anti-human immunoglobulin reagents were tested
- 30

as reagents for selective immobilization of human Fab fragments. Virtually all anti-human immunoglobulin reagents tested displayed enhanced signals compared to untreated nitrocellulose filters and it was determined
 5 that an affinity-purified polyclonal goat anti-human kappa antibody was most efficient at selectively immobilizing the BR96 Fab fragment. The non-specific signal generated by this approach was determined by overlaying filters both on areas not infected with phage
 10 and on phage plaques expressing an irrelevant antibody. The assay background was low and no difference was observed in the signal generated by either of these controls.

Using the polyclonal anti-human kappa antibody
 15 for selective immobilization, the concentration of anti-human kappa antibody for maximal binding of BR96 Fab fragment was determined. Briefly, molten 0.7% agar containing 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and the *supE* amber suppressor strain XL1-Blue
 20 (Stratagene, San Diego, CA) was overlaid on hardened bottom 1.5% agar Luria Broth medium and incubated at 37°C for 3 hours. A 3 μ l aliquot of phage stock ($\sim 10^{12}$ phage/ml) was spotted directly on the bacterial lawn and allowed to seep into the agar for 15 minutes at 25°C.
 25 The plate was then transferred to 37°C for 3-4 hours at which time large plaques were visible. Plaques were overlaid with 1 cm² nitrocellulose filters. The filters were removed and rinsed four times with 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, 2.7 mM KCl, 137 mM NaCl, pH 7.5 (PBS). To
 30 detect the selective immobilization of functional BR96 Fab, the filters were placed in PBS containing 0.772 μ g/ml horseradish peroxidase-conjugated Lewis Y antigen, 5% nonfat powdered milk, 0.2% Tween 20, 0.01% antifoam A emulsion and 0.01% thimerosal and incubated for 2 hours
 35 at 4°C. The filters were washed five times with PBS and placed in 50 mM citric acid, 100 mM Na₂HPO₄, pH 5,

containing 1 mg/ml o-phenylenediamine dihydrochloride and 0.003% hydrogen peroxide. The reaction was terminated by the addition of H_2SO_4 to a final concentration of 0.36 M, an aliquot was removed, and the OD_{490} was measured in a spectrophotometer.

As shown in Figure 1A, increasing concentrations of anti-human kappa antibody up to approximately 10 $\mu\text{g/ml}$ retained greater quantities of BR96 Fab fragment. Filters coated with more than 10 $\mu\text{g/ml}$ anti-human kappa antibody generated an enhanced, but sub-optimal signal while coating with less than 1 $\mu\text{g/ml}$ antibody did not amplify the signal appreciably. Untreated filters and filters coated with low concentrations of anti-human kappa antibody (less than 0.2 $\mu\text{g/ml}$) did not generate an assay signal above background ($A_{490} \sim 0.05$). Therefore, based on the signal obtained from filters coated with 10 $\mu\text{g/ml}$ anti-human kappa antibody ($A_{490} \sim 0.45$), the selective immobilization filter lift technique amplified the signal by at least nine-fold.

The amount of functional antibody Fab fragment expressed and selectively immobilized by the coated filters was assessed at various times from 0 to 8 hours at 25°C. As shown in Figure 1B, the signal increased from 0 to 4 hours, at which point additional transfer time did not increase the signal appreciably.

Based on these results, nitrocellulose filters were incubated 3 hours in a solution containing 10 $\mu\text{g/ml}$ of goat anti-human kappa antibody in PBS. To block unreacted sites on the nitrocellulose, the nitrocellulose filters were incubated 2 hours in a solution containing 1% bovine serum albumin (BSA) in PBS.

Filters prepared by this method were used in the studies described below.

EXAMPLE II

Immobilization of Human Fab Fragments on Solid Supports

5 Enhanced Sensitivity for Detecting Human Fab Fragments

This example demonstrates that immobilization of binding molecule populations increases the sensitivity of detecting specific binding events.

A phage library expressing BR96-reactive Fab
10 (240H2) was plated on E. coli bacterial lawns and grown at 37°C for 8 hours. Control filter lifts were generated by applying nitrocellulose filters to the bacterial lawns and incubating at 22°C for 4 hours. These control nitrocellulose filters were removed. Control filter
15 lifts, therefore, have not selectively immobilized the binding molecules expressed in phage plaques but instead have directly immobilized all secreted polypeptides and polypeptides released by lysis, including the binding molecules. To selectively immobilize human Fab
20 fragments, nitrocellulose filters coated with goat anti-human kappa antibody, prepared as in Example I, were applied to the bacterial lawns and incubated at 22°C for 4 hours. These selectively immobilized filter lifts were removed. Subsequent steps were carried out in parallel
25 with control filter lifts and selectively immobilized filter lifts. Filter lifts were blocked in 1% BSA and incubated in horse radish peroxidase-conjugated BR96 antigen (LeY antigen). Filters were developed by enhanced chemiluminescence (ECL) using procedures
30 described by the manufacturer (Amersham; Arlington Heights IL) and exposed to film for 30 seconds. The results were independent of the order the filters were applied to the bacterial lawn.

As shown in Figure 2, human Fab fragments were readily detected on selectively immobilized filter lifts (designated capture lift in Figure 2) whereas control filter lifts (designated normal lift in Figure 2) had very low levels of detectable human Fab fragments. Selectively immobilized filter lifts had much stronger signals for human antibodies that recognize the tumor antigen BR96 when compared to control filter lifts where the nitrocellulose filters are applied directly to the bacterial lawn. Thus, using a selectively immobilized filter lift to increase the concentration of the specific binding molecule of interest, in this case human Fab fragments, gave greater sensitivity for detecting the binding molecule of interest.

These results show that selectively immobilized filter lifts have increased sensitivity and decreased background binding compared to control filter lifts where binding molecules are attached directly to the filter.

EXAMPLE III

Preparation and Extraction of Biotinylated Cell Surface Antigens

This example describes a method for labeling cell surface tumor antigens. This example also describes the preparation of cell surface tumor antigen extracts that give low background binding when used for detecting selectively immobilized antibodies.

H3396 cells were grown in monolayers and chilled on ice for subsequent manipulations at 4°C. Cells were rinsed three times with PBS. After 30 minutes, cells were incubated for 2 hours with 1.5 mg/ml sulfosuccinimidyl-6-(biotinamido)hexanoate (Pierce) in PBS. Cells were rinsed once with PBS containing 50 mM glycine followed by two more rinses with PBS. Cells were

harvested by scraping in PBS containing 100 μ M [4-2-aminoethyl)-benzenesulfonylfluoride, HCl], 1 μ M pepstatin A and 10 μ M leupeptin hemisulfate. The protein concentration was determined, Triton X-100 was added to a
5 final concentration of 10:1 (w:w) and the suspension was incubated overnight at 4°C on a rotating wheel. Particulate material was removed by centrifugation at 11,000 x g for 30 minutes at 4°C followed by ultracentrifugation at 540,000 x g for 7 minutes 4°C.
10 The supernatant contains solubilized biotinylated cell surface antigens.

These results demonstrate that cell surface polypeptides can be labeled with biotin for use in screening methods. These results also demonstrate that
15 extracts devoid of interfering particulate matter, including microsomes, can be prepared for use in probing selectively immobilized filter lifts with low background binding.

EXAMPLE IV

20 Decreased Background of Selectively Immobilized Filter Lifts Relative to Control Filter Lifts

This example demonstrates that selectively immobilized filter lifts provide decreased background binding of non-specific bacterial proteins relative to
25 control filter lifts.

Phage expressing BR96-reactive Fab (240H2) were mixed with an irrelevant phage stock (IX64), which does not express any Fab, and plated on an E. coli bacterial lawn and grown for 8 hours at 37°C. Four replicate
30 filter lifts (two control filter lifts and two selectively immobilized filter lifts) were incubated sequentially with the bacterial lawns for 4 hours at 22°C. Two control filter lifts (A and B) and two

selectively immobilized filter lifts (C and D) were incubated with various reagents. To localize all plaques present on the bacterial lawn, filter lift A was incubated with rabbit anti-fd bacteriophage antibody in 1% BSA in PBS containing 0.1% sodium azide, washed three times in PBS, and subsequently incubated in alkaline phosphatase goat anti-rabbit antibody. Filters were washed six times with PBS and developed by incubation in alkaline phosphatase calorimetric reagent containing 0.1 M Tris, pH 9.5, 0.4 mM 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene)ditetrazolium chloride and 0.38 mM 5-bromo-4-chloro-3-indoxyl phosphate mono-(p-toluidinium) salt. Filter lift C was incubated with horse radish peroxidase-conjugated Lewis Y antigen (BR96 antigen). Filter lift C was developed using ECL and exposed to film for 30 seconds.

To investigate the ability of selectively immobilized filter lifts to discriminate ligands in a cell extract, H3396 cells were biotinylated and membrane extracts were prepared as described in Example III. Filter lifts B and D were incubated for 2 hours at 4°C with biotinylated cell surface antigens, prepared as described in Example III and diluted to 40 µg/ml tumor cell extract protein in 1% BSA, 1% Triton X-100, 0.145% sodium dodecyl sulfate and 0.1% sodium azide in PBS. Filter lifts B and D were washed six times with 0.1% Tween 20 in PBS and subsequently incubated with alkaline phosphatase-conjugated streptavidin. Plaques were visualized after developing in alkaline phosphatase calorimetric reagent.

As shown in Figure 3, all plaques were visualized with anti-fd bacteriophage antibody on filter lift A. Similarly, all plaques were visible when control lift B was probed with biotinylated cell surface

antigens, even though plaques derived from IX64 do not express Fab fragments. Therefore, there appears to be significant background contributed by bacterial proteins released by phage lysis. Numerous attempts to reduce the non-specific binding of tumor cell extract were made, including: (1) utilization of a variety of detergents for the solubilization of the tumor cell membranes, (2) use of more stringent wash parameters with the filters, (3) evaluation of a wide range of blocking reagents, and (4) the use of different types of matrices to capture the phage-expressed Fab. All of these approaches either failed to reduce the background or obliterated the signal entirely.

In contrast to control filters containing directly bound binding molecules and non-specific bacterial proteins, probing selectively immobilized filter lifts with purified BR96 antigen (Figure 3, filter lift C) or with a cell membrane lysate containing BR96 antigen as well as other biotinylated cell surface antigens (filter lift D) gave similar patterns of plaque detection. The use of crude, cell membrane lysate as source of BR96 antigen resulted in non-specific binding to all plaques, including those not expressing BR96-specific Fab fragments, on control filter lifts (filter lift B) whereas the same extract could be used to detect BR96-specific Fab fragments on selectively immobilized filter lifts (filter lift D). Therefore, selectively immobilized filter lifts had significantly lower background binding to bacterial proteins than control filter lifts.

These results indicate that selective immobilization of binding molecules to nitrocellulose filters produces decreased background binding to bacterial proteins. Therefore, capturing the binding molecule of interest by selectively immobilizing binding

molecules on filters increased the concentration of the binding molecule by selectively capturing the binding molecule in the bacterial plaque lysate. In addition, background was decreased by reducing the binding of non-specific bacterial proteins. Thus, the selectively immobilized filter lift method provides enhanced sensitivity and decreased background when screening binding molecules with a population of ligands.

EXAMPLE V

10 Identification of Tumor-Specific Fab Fragments Identified by Selective Immobilization on a Solid Support

This example demonstrates the identification of clones expressing tumor-specific Fab fragments.

Fab fragment libraries were generated using breast and colon tumor tissue. A large number of Fab fragments were identified with specificity for cell surface tumor antigens.

Three libraries expressing human Fab fragments, designated A2, A3 and A4, were generated from three different tumor sources. The A2 library was constructed from tumor-draining lymph node tissue obtained from a breast cancer patient. The A3 library was constructed from human colon tumor tissue. The A4 library was constructed from prostate tumor tissue. The libraries were constructed from B cells present in the tumor tissue samples. Briefly, RNA was extracted from tumor tissue using TRIZOL reagent (Gibco-BRL, Gaithersburg, MD). Following synthesis of cDNA from the RNA, the IgG heavy and kappa light chains were amplified by touchdown polymerase chain reaction (PCR). Single-stranded DNA was isolated and cloned into two vectors, IX104 and IX203. The IX104 vector contains the amber stop codon between

the immunoglobulin heavy chain and the pVIII protein.
The IX203 vector has the amber stop codon removed.

The human Fab libraries were selectively immobilized on nitrocellulose filters coated with
5 anti-human kappa antibody (prepared as in Example I).
Outlined in Table II are the results of three independent experiments screening the human Fab libraries in either the IX104 or IX203 vector against biotinylated cell surface polypeptides from the H3396 cell line (prepared
10 as described in Example III). Using the selectively immobilized filter lift technique, the frequency of positive clones varied in the three independent experiments (see Table II). As defined in these experiments, the positive clones (designated capture
15 lift-positive clones in Table II) were identified in an initial screen, picked, replated and verified in a second selectively immobilized filter lift screen.

Table II. SUMMARY OF FAB LIBRARY SCREEN WITH TUMOR CELLS

| Library | Vector | Random Clones Screened | Capture Lift-Positive Clones | Frequency | Titrate on Fixed H3396 Monolayers | Fibroblast -negative ¹ |
|---------|--------|------------------------|------------------------------|-----------|-----------------------------------|-----------------------------------|
| A2 | IX104 | 50,600 | 60 | 0.12% | 25 | 2+ |
| A3 | IX104 | 139,400 | 70 | 0.05% | 37 | 9+ |
| A3 | IX203 | 55,520 | 51 | 0.09% | 5 | 0 |
| A4 | IX203 | 13,000 | 22 | 0.17% | 6 | 2 |

¹unreactive at Fab fragment concentrations $\leq 15 \mu\text{g/ml}$

25 Positive clones identified by the selectively immobilized filter lift technique were isolated and used as a source to produce greater quantities of soluble Fab for further characterization. Briefly, XL1-Blue bacteria were grown in 2X YT medium at 37°C until the cultures
30 reached a density of 0.9 to 1.2 OD₆₀₀. Following the

addition of IPTG to a final concentration of 1 mM, the culture was infected with 10 μ l of phage from a high titer stock ($>10^{10}$ plaque forming units/ml) and incubated 1 hour at 37°C. The cultures were shifted to 25°C and
5 grown for an additional 14-16 hours. The cells were pelleted at 6000 x g for 10 minutes, resuspended in 10 ml of 50 mM Tris, pH 8.0 containing 100 μ M (4-(2-aminoethyl)-benzenesulfonylfluoride, HCl), 1 μ M pepstatin A, and 10 μ M leupeptin hemisulfate, and were broken by
10 sonic oscillation for 1 minute at 15% power output with a Branson Sonifier 450 equipped with a microtip. Following sonication, 1000 U DNaseI in 50 mM Tris, pH 8.0, was added and the lysate was incubated for 30 minutes at 25°C. The lysate was centrifuged at 12,000 x g for 30
15 minutes at 4°C and the supernatant was removed and used for purification of the Fab fragment.

The Fab fragment was purified using an affinity column constructed by coupling 20 mg of goat anti-human IgG Fab onto a 1 ml HiTrap NHS-activated column
20 (Pharmacia, Piscataway, NJ). Tween 20 was added to the bacterial cell lysate to a final concentration of 0.05% and the lysate was filtered through a 0.45 μ m filter and applied to the affinity column. The unbound fraction was collected and applied a second time. The column was
25 washed with buffer containing 50 mM Tris, pH 8.0, 0.5 M NaCl and 0.05% Tween 20 followed by 50 mM Tris, pH 8.0 buffer. The Fab was eluted with 100 mM glycine-HCl, pH 2.3, containing 0.5 M NaCl into one tenth volume 1 M Tris, pH 8.0. Fractions containing the Fab fragment were
30 applied to a PD-10 Sephadex G25-M column (Pharmacia) which had been equilibrated in PBS.

To further characterize purified Fab fragments, H3396 cell monolayers were fixed with 2% paraformaldehyde in PBS for 15 minutes at 25°C. The paraformaldehyde was
35 aspirated and cells were washed twice with PBS. Fixed

cells were incubated in blocking buffer containing 1% BSA in PBS and Fab fragments were diluted to appropriate concentrations in blocking buffer and incubated with the fixed cells for 2 hours at 25°C. The samples were
5 aspirated, the fixed cells washed three times with PBS and the cells were incubated with 0.5 µg/ml alkaline phosphatase-conjugated goat anti-human kappa light chain antibody for 1 hour at 25°C. The fixed cells were washed four times with PBS and developed with alkaline
10 phosphatase calorimetric reagent.

Approximately 40-50% of the Fab fragments identified by selective immobilization on filter lifts generated a titratable signal on fixed monolayers of H3396 tumor cells, the cell line used to generate the
15 biotinylated cell membrane lysate (see Table II). Some of the approximately 50% of Fab fragments which were not titrated on fixed monolayers are likely dependent on antigenic epitopes which were disrupted by paraformaldehyde treatment. Different techniques which
20 do not use paraformaldehyde treatment can be used to characterize these remaining Fab fragment clones.

Positive Fab fragment clones isolated by selective immobilization on a solid support, designated capture lift-positive clones in Table II, were also
25 tested for reactivity of the cloned Fab fragments against GM05658 normal human fibroblast cells. In one experiment where 50,600 original clones were screened, more than 2 clones did not react with fibroblast cells and were classified as fibroblast-negative. In a second
30 experiment where 139,400 original clones were screened, more than 9 clones were identified as fibroblast-negative clones. Thus, selective immobilization allowed the identification of human Fab fragments which specifically bind to tumor cells but not normal fibroblasts.

Using the selective immobilization filter lift technique, 81 clones expressing Fab fragments were identified as shown in Table III.

DNA was isolated from Fab clones, and DNA
5 fragments corresponding to light chain CDR regions were
sequenced using the IX104 specific primer
GCCAGTTCCAGATTTCAACTG (SEQ ID NO:1) or the IX203 specific
primer CTCTGTGACACTCTCCTGGGA (SEQ ID NO:2). DNA
fragments corresponding to heavy chain CDR regions were
10 sequenced using primer GTAGTCCTTGACCAGGCA (SEQ ID NO:3)
or AAGACCGATGGGCCCTTGGTGGAGGC (SEQ ID NO:4). Table IV
shows the amino acid sequences of three CDR regions found
in the light chain of the cloned Fab fragments. Table V
shows the nucleotide sequences of the corresponding CDR
15 regions of the light chain of cloned Fab fragments.
Table VI shows the amino acid sequences of three CDR

Table III. Tumor-specific Human Fab Clones Identified by the Selective Immobilization Filter Lift Technique

| | clone | library | family |
|----|-------|---------|----------|
| 5 | F3 | A3.104 | VH3 |
| | F13 | A3.104 | VH4 |
| | F14 | A3.104 | VH3 |
| | F15 | A3.104 | VH3, VK3 |
| | F19 | A3.104 | VH4 |
| 10 | F21 | A3.104 | VH1 |
| | F22 | A3.104 | VH6 |
| | F23 | A3.104 | VH3, VK1 |
| | F26 | A3.104 | VH3 |
| | F30 | A2.104 | |
| 15 | F31 | A2.104 | |
| | F32 | A2.104 | |
| | F33 | A2.104 | |
| | F34 | A3.104 | |
| | F35 | A3.104 | |
| 20 | F36 | A3.104 | |
| | F37 | A3.104 | |
| | F38 | A3.104 | |
| | F40 | A2.104 | |
| | F41 | A2.104 | |
| 25 | F42 | A2.104 | |
| | F46 | A2.104 | |
| | F49 | A2.104 | |
| | F50 | A2.104 | |
| | F52 | A2.104 | |
| 30 | F54 | A2.104 | |
| | F55 | A2.104 | |
| | F58 | A2.104 | |
| | F63 | A3.104 | |
| | F66 | A3.104 | |
| 35 | F67 | A3.104 | |
| | F68 | A3.104 | |
| | F69 | A3.104 | |
| | F70 | A3.104 | |
| | F72 | A3.104 | |
| 40 | F74 | A3.104 | |
| | F76 | A3.104 | |
| | F78 | A3.104 | |
| | F79 | A3.104 | |
| | F80 | A3.104 | |
| 45 | F81 | A3.104 | |
| | F84 | A3.104 | |
| | F85 | A3.104 | |
| | F86 | A3.104 | VH3, VK1 |
| | F93 | A3.203 | |

TABLE III. Tumor-specific Human Fab Clones Identified by the Selective Immobilization Filter Lift Technique

| clone | library | family |
|-------|---------|--------|
| 5 | F99 | A3.104 |
| | F104 | A3.104 |
| | F111 | A3.104 |
| | F112 | A3.104 |
| | F118 | A3.104 |
| | F126 | A3.203 |
| 10 | F129 | A3.203 |
| | F130 | A3.203 |
| | F132 | A3.104 |
| | F133 | A3.104 |
| | F134 | A3.104 |
| 15 | F135 | A3.104 |
| | F136 | A3.104 |
| | F138 | A3.203 |
| | F151 | A3.203 |
| | F158 | A3.203 |
| 20 | F160 | A3.203 |
| | F174 | A3.203 |
| | F176 | A3.203 |
| | F177 | A4.203 |
| | F184 | A4.203 |
| 25 | F186 | A4.203 |
| | F191 | A4.203 |
| | F197 | A4.203 |
| | F200 | A4.203 |
| | F202 | A4.203 |
| 30 | F203 | A4.203 |
| | F207 | A4.203 |
| | F208 | A4.203 |
| | F212 | A4.203 |
| | F214 | A4.203 |
| 35 | F217 | A4.203 |
| | F224 | A4.203 |
| | F231 | A4.203 |
| | F236 | A4.203 |
| | F238 | A4.203 |
| 40 | TA50 | A3.104 |
| | TA73 | A3.104 |

regions found in the heavy chain of the cloned Fab fragments. Table VII shows the nucleotide sequences of the corresponding CDR regions of the heavy chain of cloned Fab fragments. Each of the Fab clones identified by selective immobilization on filters contains nucleotide sequence encoding unique amino acid sequence.

Table IV. Amino Acid Sequences of CDR Regions in the Light Chains of Fab Fragments Isolated by the Selective Immobilization Filter Lift Technique

| | | | |
|---------------------|------|---------------------------|--------------|
| 5 Light Chain CDR 1 | | | |
| Clone | | | |
| | F3 | Q A S H S V F N L V G | SEQ ID NO:6 |
| | F14 | R D H S N V L A A A S W A | SEQ ID NO:8 |
| | F15 | R A S Q R V S S R F V A | SEQ ID NO:10 |
| 10 | F19 | R G L V R T L N N H L N | SEQ ID NO:12 |
| | F21 | R A S Q S V S S T N L T | SEQ ID NO:14 |
| | F22 | R A K Q N I G R W L A | SEQ ID NO:16 |
| | F23 | R A S Q D I N T Y L A | SEQ ID NO:18 |
| | F26 | R A V Q R I K Q N F G N | SEQ ID NO:20 |
| 15 | F133 | R A S Q D I N T Y L A | SEQ ID NO:22 |
| | TA50 | R A S Q T V R S S Y L A | SEQ ID NO:24 |
| | TA73 | Q A N E D I G H R L N | SEQ ID NO:26 |
| Light Chain CDR 2 | | | |
| Clone | | | |
| 20 | F3 | K S S N F T K | SEQ ID NO:28 |
| | F14 | G A S T R A T | SEQ ID NO:30 |
| | F15 | G A S S R A S | SEQ ID NO:32 |
| | F19 | A A S R L Q S | SEQ ID NO:34 |
| | F21 | A A S T L Q T | SEQ ID NO:36 |
| 25 | F22 | R A S S L E S | SEQ ID NO:38 |
| | F23 | R A S T L D I | SEQ ID NO:40 |
| | F26 | K A S R L E N | SEQ ID NO:42 |
| | F133 | R A S T L D I | SEQ ID NO:44 |
| | TA50 | G A S I R A P | SEQ ID NO:46 |
| 30 | TA73 | R S S N L K K | SEQ ID NO:48 |
| Light Chain CDR 3 | | | |
| Clone | | | |
| | F3 | Q Q Y S S T F G T | SEQ ID NO:50 |
| | F14 | Q H Y K S A P P R H T | SEQ ID NO:52 |
| 35 | F15 | Q Q S H R T P Y T | SEQ ID NO:54 |
| | F19 | Q Q S S V S R T | SEQ ID NO:56 |
| | F21 | Q H A Y A F P R T | SEQ ID NO:58 |
| | F22 | Q H Y N T N S P L T | SEQ ID NO:60 |
| | F23 | Q Q Y S R Y P L T | SEQ ID NO:62 |
| 40 | F26 | Q L Y D S H S A M Y T | SEQ ID NO:64 |
| | F133 | Q Q Y S R Y P L T | SEQ ID NO:66 |
| | TA50 | Q Q Y G R S P R A | SEQ ID NO:68 |
| | TA73 | L Q D F N Y P R T | SEQ ID NO:70 |

Table V. Nucleotide Sequences of CDR Regions in the Light Chains of Fab Fragments Isolated by the Selective Immobilization Filter Lift Technique

| | | | | | | | | | | |
|----|-------------------|-----|-----|-----|-----|-----|-----|-----|-----|--------------|
| 5 | Light Chain CDR 1 | | | | | | | | | |
| | Clone | | | | | | | | | |
| | F3 | CAG | GCC | AGT | CAC | AGT | GTT | TTT | AAC | |
| | | TTG | GTT | GGC | | | | | | SEQ ID NO:5 |
| | F14 | AGG | GAC | CAT | TCA | AAC | GTG | TTA | GCA | |
| 10 | | GCA | GCT | TCA | TGG | GCC | | | | SEQ ID NO:7 |
| | F15 | AGG | GCC | AGT | CAG | CGT | GTT | AGC | AGC | |
| | | AGA | TTC | GTA | GCT | | | | | SEQ ID NO:9 |
| | F19 | CGG | GGG | CTA | GTC | AGG | ACA | TTA | AAC | |
| | | AAC | CAT | CTA | AAT | | | | | SEQ ID NO:11 |
| 15 | F21 | AGG | GCC | AGT | CAG | AGT | GTT | AGC | AGC | |
| | | ACT | AAC | TTA | ACT | | | | | SEQ ID NO:13 |
| | F22 | CGG | GCC | AAG | CAG | AAT | ATT | GGT | CGG | |
| | | TGG | TTG | GCT | | | | | | SEQ ID NO:15 |
| | F23 | CGG | GCC | AGT | CAG | GAC | ATT | AAC | ACT | |
| 20 | | TAT | TTA | GCC | | | | | | SEQ ID NO:17 |
| | F26 | CGG | GCA | GTT | CAA | CGC | ATT | AAA | CAA | |
| | | AAT | TTT | GGG | AAT | | | | | SEQ ID NO:19 |
| | F133 | CGG | GCC | AGT | CAG | GAC | ATT | AAC | ACT | |
| | | TAT | TTA | GCC | | | | | | SEQ ID NO:21 |
| 25 | TA50 | AGG | GCC | AGT | CAG | ACT | GTT | AGG | TCC | |
| | | AGC | TAC | TTA | GCC | | | | | SEQ ID NO:23 |
| | TA73 | CAG | GCG | AAT | GAG | GAC | ATT | GGC | CAC | |
| | | CGT | TTA | AAT | | | | | | SEQ ID NO:25 |
| | Light Chain CDR 2 | | | | | | | | | |
| 30 | Clone | | | | | | | | | |
| | F3 | AAG | TCA | TCT | AAC | TTT | ACA | AAG | | SEQ ID NO:27 |
| | F14 | GGT | GCA | TCC | ACC | AGG | GCC | ACC | | SEQ ID NO:29 |
| | F15 | GGT | GCA | TCC | AGC | AGG | GCT | TCT | | SEQ ID NO:31 |
| | F19 | GCT | GCG | TCC | CGC | TTG | CAA | AGT | | SEQ ID NO:33 |
| 35 | F21 | GCT | GCA | TCC | ACT | TTG | CAA | ACT | | SEQ ID NO:35 |
| | F22 | AGG | GCG | TCC | AGT | TTA | GAA | AGT | | SEQ ID NO:37 |
| | F23 | AGG | GCG | TCT | ACT | TTA | GAC | ATT | | SEQ ID NO:39 |
| | F26 | AAG | GCG | TCT | CGT | TTA | GAA | AAT | | SEQ ID NO:41 |
| | F133 | AGG | GCG | TCT | ACT | TTA | GAC | ATT | | SEQ ID NO:43 |
| 40 | TA50 | GGT | GCA | TCC | ATC | AGG | GCC | CCT | | SEQ ID NO:45 |
| | TA73 | CGT | TCA | TCC | AAT | TTG | AAA | AAA | | SEQ ID NO:47 |

Light Chain CDR 3

Clone

| | | | |
|----|------|--|--------------|
| | F3 | CAA CAG TAT TCG AGT ACT TTC GGG ACC | SEQ ID NO:49 |
| 5 | F14 | CAG CAT TAT AAG AGC GCA CCT CCG AGG CAC ACT | SEQ ID NO:51 |
| | F15 | CAA CAG AGT CAT AGA ACC CCG TAC ACT | SEQ ID NO:53 |
| | F19 | CAA CAG AGT TCC GTT AGT CGG ACG | SEQ ID NO:55 |
| 10 | F21 | CAG CAC GCT TAC GCT TTC CCT CGG ACG | SEQ ID NO:57 |
| | F22 | CAG CAC TAT AAC ACT AAC TCT CCG CTC ACT | SEQ ID NO:59 |
| | F23 | CAA CAG TAT AGT CGT TAT CCG CTC ACT | SEQ ID NO:61 |
| 15 | F26 | CAA CTA TAT GAT AGT CAT TCC GCG ATG TAC ACT | SEQ ID NO:63 |
| | F133 | CAA CAG TAT AGT CGT TAT CCG CTC ACT | SEQ ID NO:65 |
| 20 | TA50 | CAG CAG TAT GGT AGG TCA CCT CGG GCG | SEQ ID NO:67 |
| | TA73 | CTG CAG GAT TTC AAT TAT CCC CGG ACG | SEQ ID NO:69 |

Table VI. Amino Acid Sequences of CDR Regions in the Heavy Chains of Fab Fragments Isolated by the Selective Immobilization Filter Lift Technique

| | | | |
|----|-------------------|---------------------------------------|---------------|
| 5 | Heavy Chain CDR 1 | | |
| | Clone | | |
| | F3 | S Y S M N | SEQ ID NO:72 |
| | F14 | D Y A I H | SEQ ID NO:74 |
| | F15 | T Y W M S | SEQ ID NO:76 |
| 10 | F19 | S S L W W S | SEQ ID NO:78 |
| | F21 | R Y Y M N | SEQ ID NO:80 |
| | F22 | S N R A T W N | SEQ ID NO:82 |
| | F23 | S Y A M N | SEQ ID NO:84 |
| | F26 | F S N Y D L N | SEQ ID NO:86 |
| 15 | F133 | S A W L S | SEQ ID NO:88 |
| | TA50 | S Y W I S | SEQ ID NO:90 |
| | TA73 | T Y G L S | SEQ ID NO:92 |
| | Heavy Chain CDR 2 | | |
| | Clone | | |
| 20 | F3 | Y F S S S G T T I Y Y A D S V K G | SEQ ID NO:94 |
| | F14 | F I R S E A F G G T A D Y A A S V K G | SEQ ID NO:96 |
| | F15 | N I N Q D G S E K Y Y V D S V E G | SEQ ID NO:98 |
| 25 | F19 | E M Y H S G S T K Y N P P S R V | SEQ ID NO:100 |
| | F21 | I I N P S G D R T R Y A Q K F Q G | SEQ ID NO:102 |
| 30 | F22 | R T Y Y R S E W F N D Y A V F V Q S | SEQ ID NO:104 |
| | F23 | Y I S S R G S T I Y Y A D S V K G | SEQ ID NO:106 |
| | F26 | T F T G S G D N T Y Y A D S V K G | SEQ ID NO:108 |
| 35 | F133 | N I K P D G S D K Y Y V D S V K G | SEQ ID NO:110 |
| | TA50 | R I D P S D S Y T N Y S P S F Q G | SEQ ID NO:112 |
| 40 | TA73 | W I K P Y N G Y T K Y A Q R L Q D | SEQ ID NO:114 |

Heavy Chain CDR 3
Clone

| | | | |
|----|------|---------------------------------------|---------------|
| | F3 | G R K T S R Y T L R R F R S I W F D Y | |
| | | | SEQ ID NO:116 |
| 5 | F14 | V G R P G E D F | SEQ ID NO:118 |
| | F15 | D P P R Y F G S G S W E | SEQ ID NO:120 |
| | F19 | A S Y T N K W T P I A Y F E Y | SEQ ID NO:122 |
| | F21 | P L V Q R D T F Q E | SEQ ID NO:124 |
| | F22 | V P Q V E V P Y | SEQ ID NO:126 |
| 10 | F23 | F M N R D G P P K R V F D Y | SEQ ID NO:128 |
| | F26 | G V S T M V R G L T | SEQ ID NO:130 |
| | F133 | A G P L R I R Y D G F D F | SEQ ID NO:132 |
| | TA50 | G R K T S R Y T L R R S E A S G F D Y | SEQ ID NO:134 |
| 15 | TA73 | D A H R G L V G A T A N F H Y | SEQ ID NO:136 |

Heavy Chain CDR 1
Clone

| | | | |
|--------------------------------|------|---|---------------|
| | F3 | TCC TAT AGC ATG AAC | SEQ ID NO:71 |
| | F14 | GAC TAT GCT ATA CAT | SEQ ID NO:73 |
| | F15 | ACC TAT TGG ATG AGC | SEQ ID NO:75 |
| 10 | F19 | AGT CTT TGG TGG AGT | SEQ ID NO:77 |
| | F21 | AGG TAT TAT ATG AAC | SEQ ID NO:79 |
| | F22 | AGC AAT CGT GCT ACG TGG AAC | SEQ ID NO:81 |
| | F23 | AGC TAT GCC ATG AAT | SEQ ID NO:83 |
| | F26 | TTT AGT AAT TAT GAT CTG AAC | SEQ ID NO:85 |
| 15 | F133 | AGC GCC TGG CTG AGC | SEQ ID NO:87 |
| | TA50 | AGC TAC TGG ATC AGC | SEQ ID NO:89 |
| | TA73 | ACG TAT GGT CTC AGC | SEQ ID NO:91 |
| Heavy Chain CDR 2 Clone | | | |
| 20 | F3 | TAT TTT AGT AGT AGT GGG ACC ACC ATA TAT TAC GCA GAC TCT GTG AAG GGC | SEQ ID NO:93 |
| | F14 | TTC ATT AGA AGT GAG GCC TTT GGT GGG ACA GCA GAT TAC GCC GCG TCT | |
| 25 | | GTG AAA GGC | SEQ ID NO:95 |
| | F15 | AAC ATA AAC CAA GAT GGA AGT GAG AAA TAC TAT GTG GAC TCT GTG GAG GGC | SEQ ID NO:97 |
| | F19 | GAA ATG TAT CAT AGT GGG AGC ACC AAA TAC AAC CCT CCC TCA AGA GTT | SEQ ID NO:99 |
| 30 | F21 | ATT ATC AAC CCT AGT GGT GAT AGA ACA AGA TAC GCA CAG AAG TTC CAG GGC | SEQ ID NO:101 |
| | F22 | AGG ACA TAC TAC AGG TCC GAG TGG TTT AAT GAT TAT GCA GTA TTT GTG CAA AGT | SEQ ID NO:103 |
| 35 | F23 | TAC ATT AGT AGT CGT GGT AGT ACC ATA TAC TAC GCT GAC TCT GTG AAG GGC | SEQ ID NO:105 |
| 40 | F26 | ACT TTT ACT GGT AGT GGT GAC AAC ACA TAC TAC GCA GAC TCC GTG AAG GGC | SEQ ID NO:107 |
| | F133 | AAC ATA AAG CCA GAT GGA AGT GAC AAA TAT TAT GTG GAC TCT GTG AAG GGC | SEQ ID NO:109 |
| 45 | TA50 | AGG ATT GAT CCT AGT GAC TCT TAT ACC AAC TAC AGC CCG TCC TTC CAA GGC | SEQ ID NO:111 |
| | TA73 | TGG ATC AAG CCT TAC AAT GGT TAT ACA AAG TAT GCA CAG CGC CTC CAG GAT | SEQ ID NO:113 |
| 50 | | | |

Heavy Chain CDR 3
Clone

| | | | |
|----|------|---------------------------------|---------------|
| | F3 | GGA AGG AAG ACC TCT CGA TAT ACT | |
| | | TTG CGA CGT TTC AGA AGT ATC TGG | |
| 5 | | TTT GAC TAC | SEQ ID NO:115 |
| | F14 | GTC GGG CGC CCG GGT GAG GAC TTT | SEQ ID NO:117 |
| | F15 | GAT CCC CCA CGA TAC TTT GGT TCG | |
| | | GGG AGT TGG GAA | SEQ ID NO:119 |
| | F19 | GCC AGT TAT ACC AAC AAA TGG ACC | |
| 10 | | CCC ATT GCC TAC TTT GAA TAC | SEQ ID NO:121 |
| | F21 | CCC CTG GTT CAA CGG GAT ACT TTC | |
| | | CAG GAA | SEQ ID NO:123 |
| | F22 | GTC CCA CAG GTA GAG GTG CCT TAT | SEQ ID NO:125 |
| | F23 | TTT ATG AAT AGA GAT GGC CCC CCG | |
| 15 | | AAG AGG GTG TTT GAC TAC | SEQ ID NO:127 |
| | F26 | GGG GTT TCG ACT ATG GTT CGG GGA | |
| | | CTG ACC | SEQ ID NO:129 |
| | F133 | GCT GGC CCA CTC CGT ATC CGG TAT | |
| | | GAT GGT TTT GAT TTT | SEQ ID NO:131 |
| 20 | TA50 | GGA AGG AAG ACC TCT CGA TAT ACT | |
| | | TTG CGA CGT TCA GAA GCA TCT GGT | |
| | | TTC GAC TAC | SEQ ID NO:133 |
| | TA73 | GAT GCC CAT CGC GGT CTA GTG GGA | |
| | | GCT ACA GCA AAC TTT CAC TAC | SEQ ID NO:135 |
| 25 | | | |

Using the selective immobilization filter lift technique, a large number of human Fab fragments produced in an expression library were identified. The technique allowed the screening of a large number of clones. DNA sequencing of selected numbers of isolated Fab clones revealed that a variety of genetically distinct Fab fragments were identified.

These results show that the selective immobilization filter lift technique allows identification of a large number of distinct human Fab fragments.

EXAMPLE VI

Characterization of Tumor Cell Specific Human Fab
Fragments Identified by Selective Immobilization on a
Solid Support

5 This example demonstrates cell specific reactivity of Fab clones with respect to fixed and live tumor cell lines.

 Fab fragments identified using the selective immobilization filter lift technique were purified as
10 described in Example V. Various cell lines were fixed in paraformaldehyde as described in Example V and incubated with increasing concentrations of purified Fab fragments (2.5×10^{-1} to 3×10^1 $\mu\text{g/ml}$). Unbound Fab was removed and fixed cells were incubated with alkaline phosphatase-
15 conjugated anti-human kappa antibody as described in Example V. Following incubation with color reagents, the OD_{560} was measured.

 In Figure 4A, the titration curve of purified Fab F15 is shown on two breast carcinoma cell lines
20 (H3396 and H3464) and GM05658 normal human fibroblasts. Fab F15 shows specific binding to both breast cell lines but not to normal fibroblasts. In Figure 4B, the titration curve of Fab F133 is shown with the same two breast cell lines and GM05658 normal human fibroblasts.
25 Fab F133 shows specific binding for the H3396 cells used for making a screening extract. However, Fab F133 does not bind to the second breast cell line or to the normal fibroblasts.

 Purified Fab fragments were also characterized
30 with respect to binding to live tumor cells by FACS analysis on a Becton Dickinson FACSsort system. Purified Fab F3 was compared to a non-specific Fab fragment on H3396 cells (see Figure 5A). Incubation of H3396 cells

with Fab F3 caused a shift in H3396 cells, indicating reactivity with a cell surface antigen, compared to cells incubated with non-specific Fab. The peak of Fab F3 shifted cells is sharp, indicating that the population of
5 H3396 cells are relatively homogeneous in their expression of antigen.

A similar FACS analysis was performed with Fab F15. As shown in Figure 5B, Fab F15 resulted in a larger shift in H3396 cells relative to non-specific Fab than
10 was observed with Fab F3, indicating that Fab F15 reacts with a cell surface antigen. The Fab F15 shifted peak is broader than that observed with Fab F3, consistent with a more heterogeneous expression of the Fab F15 antigen on H3396 cells.

15 A FACS analysis was also performed with Fab F40. FAB F40 was identified using membrane extracts of the breast cell line H3396. Interestingly, Fab F40 is cross-reactive with the colon carcinoma cell line H3719 (see Figure 5C). Fab F40 results in a shift in H3719
20 cells relative to control non-specific Fab, indicating that Fab F40 recognizes a cell surface antigen on the colon carcinoma cell line. The Fab F40 peak is somewhat broad, indicating that expression of the F40 antigen is more heterogeneous. Thus, Fab F40 recognizes a cell
25 surface antigen on two different types of tumor cells.

The selective immobilization filter lift technique allowed isolation of a large number of human Fab fragments with specificity for fixed and live tumor cells. While some Fab fragments are specific for breast
30 carcinoma cells or are specific for a particular breast cell line, other Fab fragments cross react with a colon carcinoma cell line. These Fab fragments displayed specific binding on tumor cells but did not bind to normal fibroblasts.

These results indicate that the selective immobilization filter lift technique allows identification of human Fab fragments with specificity for tumor cells.

- 5 All journal article and references provided above, in parentheses or otherwise, whether previously stated or not, are incorporated herein by reference in their entirety.

- 10 Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the claims.